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# Phosphorylation of human polyomavirus BK agnoprotein at Ser-11 is mediated by PKC and has an important regulative function

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ABSTRACT

The human polyomavirus BK (BKV) genome encodes the capsid proteins VP1 to VP3 and the three regulatory proteins, large and small tumor-antigen and the agnoprotein. Agnoprotein is a phospho-protein, but phosphorylation sites, protein kinases that mediate phosphorylation, and the biological importance of phosphorylation for the life-cycle of BK virus remain unknown. Here, we show that protein kinase C phosphorylates BKV agnoprotein at serine-11. Replacing serine-11 by either non-phosphorylable alanine or phospho-mimicking aspartic acid reduced the ability of these mutants to propagate compared to wildtype virus. Moreover, both these mutants displayed altered expression of viral proteins, which resulted from changed transrepressive property and stability of the mutated agnoprotein. Our results indicate that BKV propagation is controlled by phosphorylation of the agnoprotein and may suggest that specific inhibition of protein kinases may be used as a therapeutic strategy to hamper BK virus infection.



## Introduction

*Polyomaviridae* are non-enveloped viruses with an icosahedral capsid of about 40–45 nm in diameter and they contain a circular double-stranded DNA molecule of approximately 5000 bp. Members of this family have been identified in several organisms, including birds, bovine, rodents, monkeys and human (Eash et al., 2006). The first human polyomaviruses were originally isolated in 1971 by two independent research groups. Gardner and coworkers isolated BK virus (BKV) in the urine of a renal transplant patient with the initials B.K., while Padgett and colleagues characterized a virus in the brain of a Hodgkin's lymphoma patient with initials J.C. who suffered from progressive multifocal leukencephalopathy. The Rhesus macaques virus SV40 was accidentally introduced into the human population via contaminated vaccines, but recent studies reveal antibodies against SV40 and SV40 nucleic acid sequences in individuals that had never received such vaccines nor had contact with monkeys (reviewed in Moens et al., 2007a,b). Therefore SV40 may also be considered as a human virus (Moens et al., 2007a,b). All these three human viruses have been associated with human pathologies. JCV is the etiological agent of PML (Khalili and White, 2006), while BKV is associated with nephropathy in renal transplant patients, hemorrhagic cystitis in bone marrow recipients (Hirsch, 2005; Hirsch and Steiger, 2003), and the autoimmune disease systemic lupus erythematosus (Van Ghelue et al., 2003). SV40 is considered to play a causative role in mesothelioma (Pass et al., 2004). Recently, three novel human polyomaviruses have been described. KI and WU were discovered in respiratory secretions (Allander et al., 2007; Gaynor et al., 2007), while Merkel cell polyomavirus (MCV) was described as the likely causative agent of Merkel skin cancer (Feng et al., 2008).

The JCV, BKV and SV40 genomes possess a tripartite functional organization with the noncoding control region (NCCR) that separates the early gene and late gene region. The early region encodes the regulatory proteins large tumor-antigen (LT-ag) and small tumor-antigen (st-ag), while the late region encodes the structural capsid proteins VP1, VP2 and VP3 as well as the regulatory agnoprotein (reviewed by Moens and Rekvig, 2001). For SV40, a fourth late protein, VP4, was recently identified as a protein involved in lytic death of host cell and release of viral progeny (Daniels et al., 2007).

The agnoprotein of JCV, BKV and SV40 has a high degree of sequence conservation, especially in the N-terminal half of the protein where 31 or 25 amino acids out of 37 is identical between BKV and JCV or BKV and SV40, respectively. The agnoprotein has been shown to reside predominantly in the cytosol and in the perinuclear region in association with the outer nuclear membrane, but a minor fraction can also be detected in the nucleus of SV40- and JCV-infected cells (Khalili et al., 2005). Studies in JCV and/or SV40 have suggested that agnoprotein is involved in nuclear egress (Okada et al., 2005), gene

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expression, viral release, virion assembly and maturation, reviewed in (Khalili et al., 2005). The function of BKV agnoprotein in the viral life cycle has not been investigated so far.

Phosphorylation is a common post-translational modification of cellular proteins and is performed by protein kinases which transfer phosphates to serine, threonine and tyrosine residues. Phosphorylation of a target protein may modify its subcellular localization, stability, activity, and/or contact with interaction partners (Whitmarsh and Davis, 2000). The human genome encodes more than 500 different protein kinases, which differ in their substrate specificity, subcellular localization, regulation and expression. Among the best studied serine–threonine kinases are protein kinases A (PKA), C (PKC) and D (PKD). The PKC family consists of several isoforms which are classified into three major subfamilies: the classical PKC (α, β and γ), novel PKC (δ, ε, η, and θ) and atypical PKC (ξ and ι) (Webb et al., 2000). The PKD family comprises PKD1, PKD2 and PKD3 (Rozengurt et al., 2005), while PKA exists as a tetramer composed of two regulatory and two catalytic subunits. Different isoforms of these regulatory and catalytic subunits occur in the cell and they can assemble in different combinations, generating different types of PKA with different properties (Skalhegg and Tasken, 2000; Tasken and Aandahl, 2004). The different protein kinases are activated by different stimuli, resulting in a fine-tuned mechanism allowing a specific substrate to be phosphorylated as a consequence of a particular environmental change (Ubersax and Ferrell, 2007).

The agnoprotein of all well-known primate polyomaviruses has been found to be phosphorylated *in vivo* (Khalili et al., 2005). A recent study revealed that Ser-7, Ser-11 and Thr-21 are phospho-acceptor sites in JCV agnoprotein. Mutation of these sites into non-phosphorylable alanine resulted in disturbed viral propagation (Sariyer et al., 2006). The Ser-7 and -11 residues are conserved in SV40 and BKV, while Thr-21 is conserved in BKV, but is replaced by Ser in SV40. Whether these sites are phosphorylated in SV40 or BKV and which kinases are involved in mediating these phosphorylations remain to be established. Moreover, the role of phosphorylation of agnoprotein in viral propagation of BKV and SV40 has not been elucidated.

The aim of this study was to identify the sites and protein kinases that mediate phosphorylation of BKV agnoprotein, and to scrutinize the biological implications of this modification. In this study, we show that agnoprotein of BKV can be phosphorylated at Ser-11 by PKA, and also at additional sites by PKC and PKD *in vitro*. Furthermore, we demonstrate that PKC can mediate phosphorylation of agnoprotein at Ser-11 *in vivo*. We provide evidence that phosphorylation of agnoprotein at Ser-11 fluctuated during viral propagation indicating an important role in the viral life cycle. Indeed, mutation of Ser-11 residue resulted in changed viral propagation with differences in late gene expression, compared to wildtype BKV and an agno-deficient BKV variant. Moreover, we show that agnoprotein and agnoprotein with mutation at Ser-11 residue differ in their transrepression ability of late viral promoter and also in their stability, which might partially explain the changed gene expression among the BKV variants.

## Results

### *BKV agnoprotein can be phosphorylated by PKA, PKC and PKD in vitro*

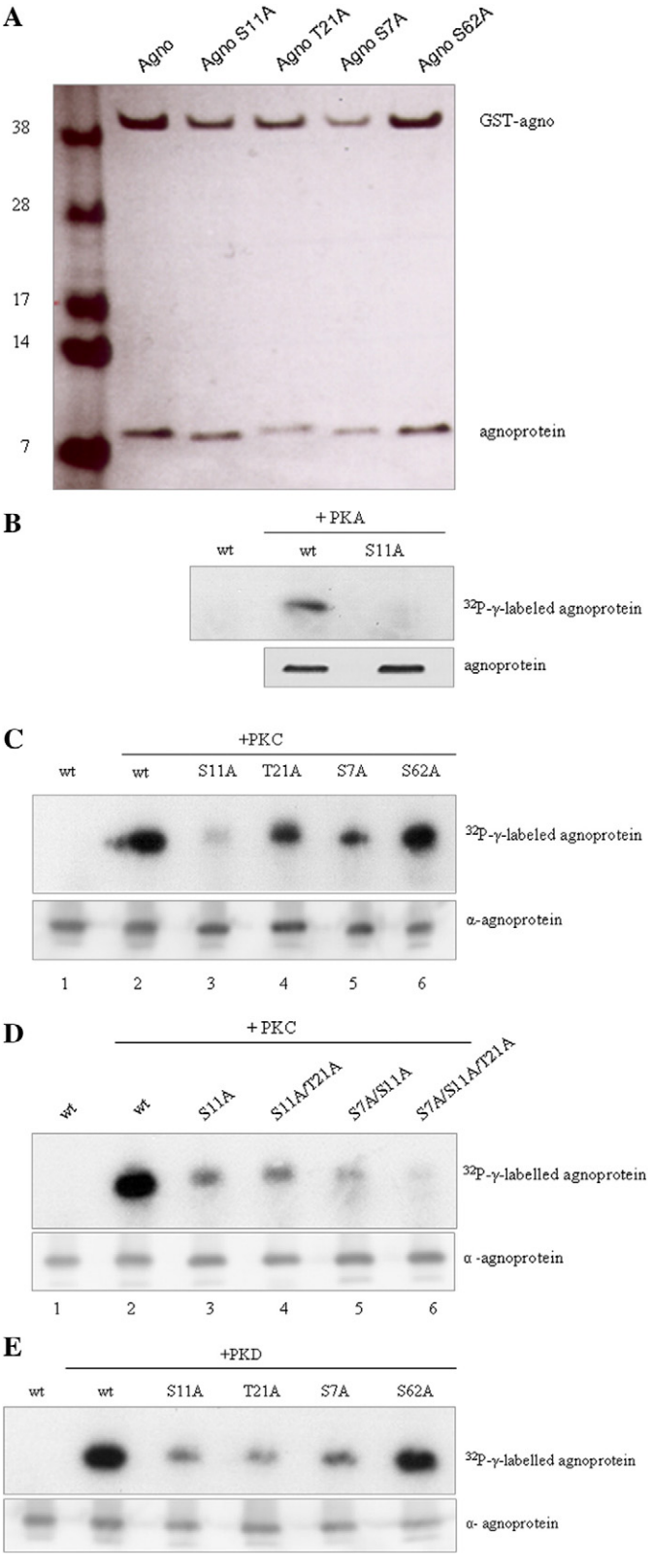
In order to determine putative phospho-acceptor sites in BKV agnoprotein, we first performed a computer-aided motif search analysis of the protein. The analysis predicted Ser-7, Ser-11, Thr-21, Ser-52, Ser-62 and Ser-66 as putative phospho-acceptor sites (Blom et al., 1999). An amino acid sequences alignment of BKV, JCV and SV40 revealed that Ser-7, Ser-11, and Ser-62 are conserved, while Thr-21 in BK and JC is replaced by serine in SV40. In order to determine which sites of agnoprotein could be phosphorylated *in vitro*, we purified bacterial expressed GST-agno and GST-agno variants where one or several of the conserved phospho-acceptor sites were mutated into the non-phosphorylable alanine. The GST-tag was enzymatically removed and the samples were analyzed on SDS PAGE followed by Coomassie staining (Fig. 1A and results not shown). Agnoprotein of all variants were successfully purified (~7 kDa fragment), but also remaining fusion protein (~38 kDa fragment) is detected in all samples.

PKA has previously been suggested to be involved in phosphorylation-regulated subcellular localization of JCV agnoprotein (Okada et al., 2001), and we therefore investigated the ability of PKA to phosphorylate agnoprotein or agnoprotein where Ser-11 was mutated into the non-phosphorylable alanine (S11A) *in vitro*. Our results revealed that PKA clearly phosphorylated wildtype agnoprotein devoid of the GST moiety, while agnoproteinS11A was not phosphorylated (Fig. 1B, upper panel). The amounts of agnoprotein used in the kinase assay were run on SDS PAGE in order to evaluate the protein level. As shown in Fig. 1B (lower panel) the levels of agnoprotein used in the assay are comparable, confirming that the lack of autoradiography bands for agnoproteinS11A is because of the mutation in the phospho-acceptor site for PKA and not the absence of protein. These results imply that Ser-11 is the only phospho-acceptor site for PKA *in vitro*.

PKC was recently shown to phosphorylate JCV agnoprotein (Sariyer et al., 2006), and this prompted us to test whether this kinase also could phosphorylate agnoprotein of BKV. Wildtype or mutated BKV agnoprotein where Ser-7, Ser-11, Thr-21 or Ser-62 was replaced by the non-phosphorylable alanine were used in an *in vitro* kinase assay, and the phosphorylated proteins were separated on SDS PAGE and either visualized by autoradiography (Fig. 1C, upper panel) or by immunoblot with antibodies against agnoprotein in order to compare the amount of proteins used in the assay (Fig. 1C, lower panel). PKC clearly mediated phosphorylation of agnoprotein (compare lane 1 and 2, upper panel). Moreover, PKC phosphorylated agnoprotein and agnoproteinS62A approximately equally, which indicates that Ser-62 is not an *in vitro* phospho-acceptor site for PKC (compare lane 2 and 6, upper panel). Substitution of Thr-21 into Ala reduced PKC-mediated phosphorylation, while S7A and S11A mutations strongly or almost completely abrogated PKC-mediated phosphorylation, indicating that all three sites may be phospho-acceptor sites for PKC *in vitro*, although with different affinity. We therefore extended these studies and tested whether double and triple mutants of agnoprotein could be phosphorylated by PKC. The stoichiometry of the phosphorylated double mutant S11A/T21A and S7A/S11A was slightly less compared to the single mutant agnoproteinS11A, while PKC-induced phosphorylation was completely abolished in the S11A/T21A/S7A mutant (Fig. 1D). The levels of agnoprotein used in the assays were similar as determined by western blot, confirming that the lack of phosphorylation is not due to less protein (Fig. 1C and D, lower panels). These results indicate that Ser-7, Ser-11 and Thr-21 can be phosphorylated by PKC *in vitro*.

PKD has an unique substrate specificity, with a consensus sequence LXRXXT*/S*, where T*/S* denotes the target threonine and serine for phosphorylation, respectively (Nishikawa et al., 1997; Hutti et al., 2004; Doppler et al., 2005). Interestingly, agnoprotein Ser-11 is contained within the PKD consensus motif LSRRPS*. Therefore we monitored by *in vitro* kinase assay whether agnoprotein forms a PKD substrate. The phosphorylation pattern of single mutants of agnoprotein indicated that the phospho-acceptor sites for PKD are Ser-7, Ser-11 and Thr-21, but not Ser-62 (Fig. 1E, upper panel). The amount of proteins used in all the assays was comparable (Fig. 1E, lower panel), again confirming that the lack of phosphorylation was due to the mutation rather than the absence of protein in the samples. Mutation of the three phospho-acceptor sites into alanine completely abolished PKD-mediated phosphorylation of agnoprotein (results not shown). These results show that Ser-7, Ser-11 and Thr-21 can be phosphorylated by PKD *in vitro*.

As Ser-11 was the only site that became phosphorylated by all three kinases *in vitro*, and mutation of this site had the most pronounced effect on the phospho-agnoprotein levels, we generated affinity-purified phospho-Ser-11 specific antibodies. The specificity of the antibody was checked on immunoblot containing *in vitro* phosphorylated agnoprotein or agnoproteinS11A. As seen in Fig. 2A the phospho-Ser-11-specific antibody specifically recognized the *in vitro* phosphorylated agnoprotein



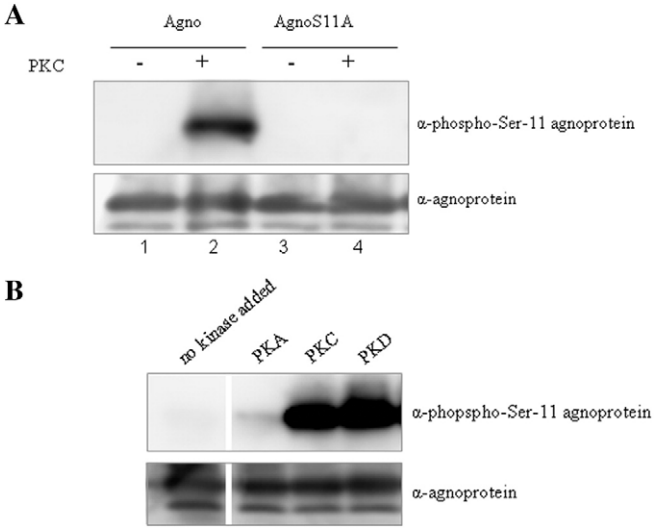




**Fig. 2.** The phospho-specific antibody specifically recognizes agnoprotein phosphorylated at Ser-11. (A) Purified agnoprotein or agnoprotein with the mutation S11A were incubated in the presence or absence of PKC. An immunoblot was performed using phospho-Ser-11 specific antibodies (upper panel). To verify equal loading and blotting of the samples, the membrane was stripped and re-incubated with anti-agno antibodies (lower panel). The results are representative for at least three independent experiments. (B) Purified agnoprotein was used in a cold *in vitro* kinase assay with active PKA, PKC or PKD. An immunoblot was performed with phospho-Ser-11 specific antibodies (upper panel). To verify equal loading and blotting of the samples, the membrane was stripped and re-incubated with anti-agno antibodies (lower panel).

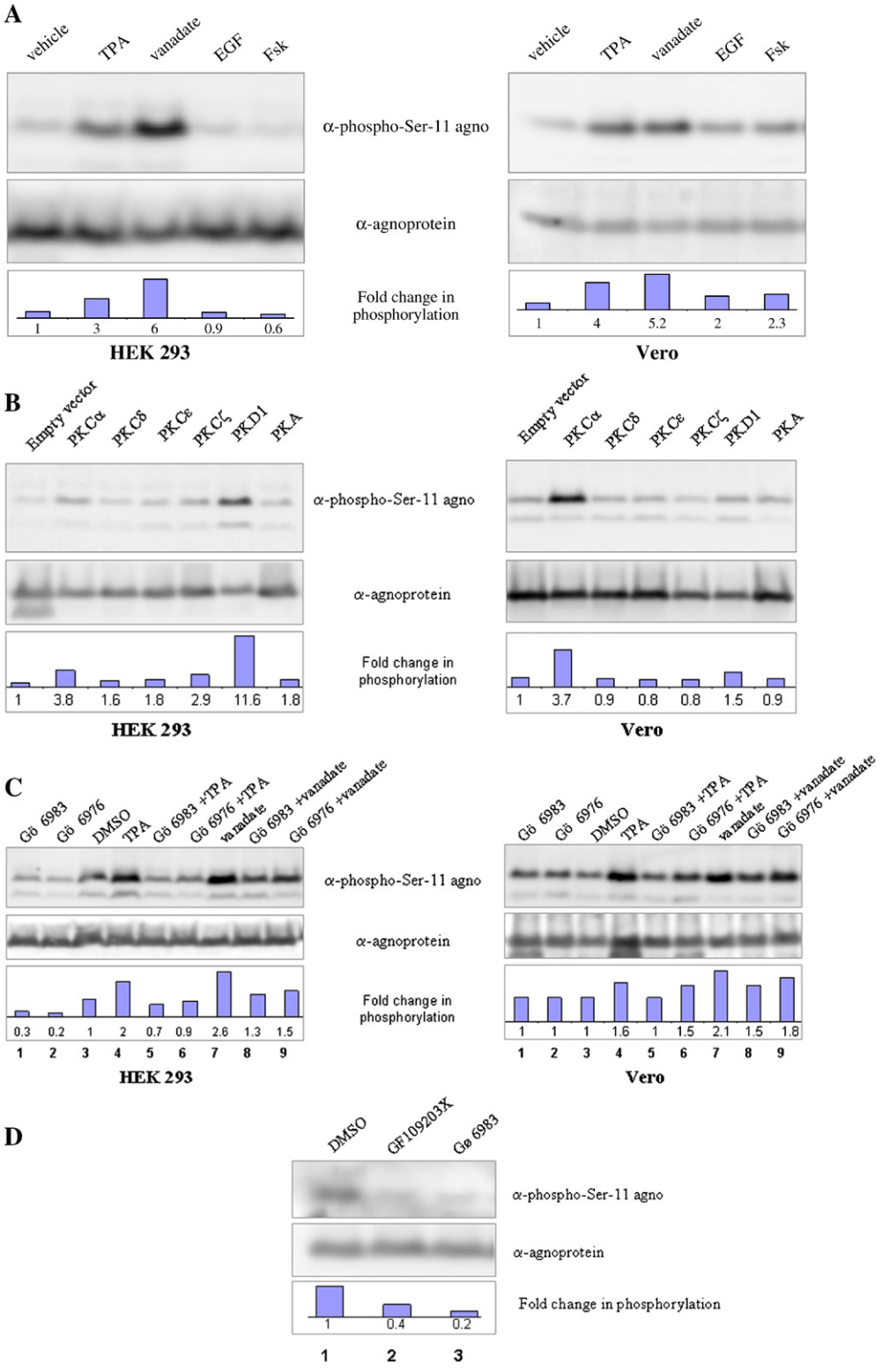
(lane 2), but not agnoproteinS11A (lane 4), nor unphosphorylated agnoprotein variants (lane 1 and 3). The lower panel shows that the amount of agnoprotein used in the experiment was comparable for all samples. This result clearly demonstrates that the antibody against phospho-Ser-11 agnoprotein specifically recognizes agnoprotein phosphorylated at Ser-11. Finally, the PKA-, PKC- and PKD-phosphorylated or non-phosphorylated agnoprotein were separated on SDS PAGE and immunoblotted with phospho-Ser-11 antibodies (Fig. 2B, upper panel). The loading was controlled by stripping the membrane prior to addition of antibodies against agnoprotein (Fig. 2B, lower panel). By use of this phospho-specific antibody we confirmed the PKA-, PKC- and PKD-mediated phosphorylation of agnoprotein at Ser-11, although phosphorylation by PKA was only marginal compared to PKC and PKD (Fig. 2B).

**Fig. 1.** PKA, PKC and PKD phosphorylate BKV agnoprotein *in vitro*. (A) Purified agnoprotein or agnoprotein single mutants in putative phosphorylation sites (Serine-7, Serine-11, Threonine-21, and Serine-62) were separated on SDS PAGE and stained with Coomassie blue. The molecular mass marker (in kDa) is shown in the left lane. (B) Bacterial expressed agnoprotein or agnoprotein where Ser-11 is mutated into alanine were purified and used in *in vitro* kinase assay with active PKA in the presence of $^{32}$P-γ-ATP. Lane 1 represents *in vitro* kinase of purified agnoprotein in the absence of PKA The *in vitro* phosphorylated proteins were separated by SDS PAGE and visualized by autoradiography. The lower panel shows Coomassie blue staining of the purified agnoprotein variants used in the kinase assay. (C) Bacterial expressed agnoprotein or agnoprotein single mutants in putative phosphorylation sites (serine-11, threonine-21, serine 7, and serine 62) were used in *in vitro* kinase assay with active PKC in the presence of $^{32}$P-γ-ATP. Lane 1 represents *in vitro* kinase of purified agnoprotein in the absence of PKC. The *in vitro* phosphorylated proteins were separated by SDS PAGE and visualized by autoradiography. The lower panel shows immunoblot with anti-agno antibodies visualizing the amount proteins used in the *in vitro* kinase assay. (D) Bacterial expressed agnoprotein, agnoproteinS11A, agnoproteinS11A/T21A, agnoproteinS7A/S11A and agnoproteinS7A/S11A/T21A were used in *in vitro* kinase assay with active PKC in the presence of $^{32}$P-γ-ATP. Lane 1 represents *in vitro* kinase of purified agnoprotein in the absence of PKC. The *in vitro* phosphorylated proteins were separated on SDS PAGE and visualized by autoradiography. The lower panel shows immunoblot with anti-agno antibodies of the amount proteins used in the *in vitro* kinase assay. (E) Bacterial expressed agnoprotein or agnoprotein single mutants in putative phosphorylation sites (Serine-7, Serine-11, Threonine-21, and Serine-62) were used in *in vitro* kinase assay with active PKD in the presence of $^{32}$P-γ-ATP. Lane 1 represents *in vitro* kinase of purified agnoprotein in the absence of PKD. The *in vitro* phosphorylated proteins were separated on SDS PAGE and visualized by autoradiography. The lower panel shows immunoblot with anti-agno antibodies of the amount proteins used in the *in vitro* kinase assay.

In conclusion, our *in vitro* studies demonstrate that Ser-11 of agnoprotein can be phosphorylated by PKA, PKC and PKD, while PKC and PKD can additionally phosphorylate agnoprotein at Ser-7 and Thr-21.

*PKC α and PKD are able to phosphorylate agnoprotein at Ser-11 in HEK293 and Vero cells*

Our *in vitro* kinase results indicate that PKA, PKC and PKD are able to phosphorylate agnoprotein at serine 11. Therefore, we examined whether extracellular stimuli which activate these kinases may trigger phosphorylation of agnoprotein at Ser-11 *in vivo*. Thereto, we transfected HEK293 and Vero cells with expression plasmid for agnoprotein and exposed the cells to the following stimuli: 12-*O*-tetradecanoylphorbol-13-acetate (TPA); mimics diacylglycerol-mediated signaling pathways resulting in activation of among others classical and novel PKCs and PKD (Brose et al., 2004), vanadate (induces oxidative stress, and activates among others also PKD (Rozengurt et al., 2005), epidermal growth factor (EGF) (activates among others atypical PKCs



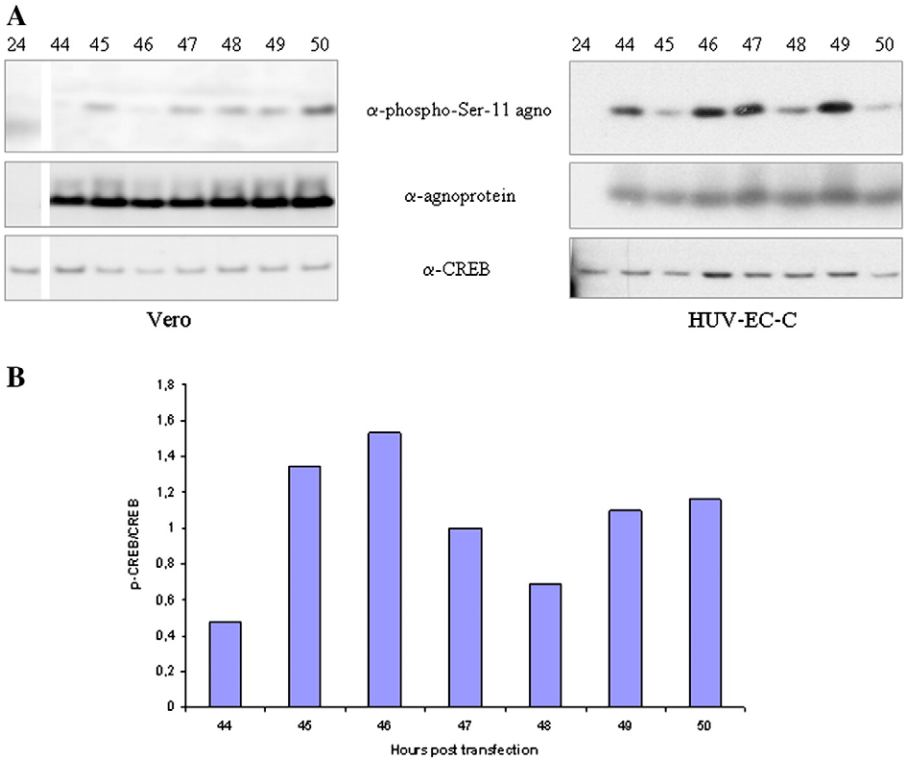



**Fig. 4.** Phospho-Serine-11 phosphorylation levels fluctuate during viral propagation. (A)Vero cells were transfected with BKV Dunlop DNA (left panel) and HUV-EC-C cells were infected with BKV Dunlop at MOI=0.4 (right panel). Total cell extracts were harvested in a time-dependent manner and phospho-serine-11 agnoprotein levels were monitored by immunoblot with phospho-Ser-11 specific antibodies. The expression of agnoprotein was evaluated antibodies against agnoprotein, and the loading was controlled by use of antibodies against endogenous cellular CREB. The results are representative for three independent experiments. (B) The phospho-CREB expression was determined in the extracts of Vero cells presented in (A). The ratio phospho-CREB versus CREB was calculated and is presented graphically.

(Webb et al., 2000) or forskolin (fsk); adenylate cyclase activator which enhances intracellular cAMP levels and PKA activity (Walsh et al., 1968). The cell lysates were separated on SDS PAGE and an immunoblot was performed using phospho-Ser-11 specific antibodies (Fig. 3A, upper panel). Equal loading was verified by the use of antibodies against agnoprotein (Fig. 3A, lower panel) The results showed that stimulation with TPA and vanadate, but not EGF nor forskolin, provoked phosphorylation of agnoprotein at Ser-11 in HEK293 (Fig. 3A upper panel). Stimulation of Vero cells gave similar results, except that in this cell line forskolin and EGF also weakly, but reproducibly induced increased phosphorylation of agnoprotein at Ser-11. These data suggest that only protein kinases activated by TPA and vanadate can readily mediate phosphorylation of agnoprotein at Ser-11 in both cell lines.

To further explore the involvement of PKA, PKC and PKD in mediating phosphorylation of agnoprotein, we co-transfected HEK293 and Vero cells with expression plasmids for agnoprotein and constitutive active variants of classical PKCα, novel PKC ε, atypical PKCζ, PKD1 or PKA. Of the tested kinases, PKCα and PKD were most efficient in mediating phosphorylation of agnoprotein at Ser-11 in both cell lines. In contrast to PKCα and PKD, the presence of constitutive variants of PKA, PKCε and PKCζ did not result in augmented Ser-11 phosphorylation in both cell lines (Fig. 3B).

Our results demonstrate that certain isoforms of PKC and PKD can mediate phosphorylation of agnoprotein at Ser-11 *in vivo*. However, even though TPA and vanadate are well-known activators of PKC and PKD, they may in addition also activate other signaling pathways (Brose et al., 2004; Fantus and Tsiani, 1998). Therefore, we wanted to confirm the contribution of these kinases in phosphorylation of agnoprotein and assayed phospho-Ser-11 agnoprotein levels in extracts of TPA- or vanadate-stimulated HEK293 and Vero cells in the absence or presence of the inhibitors Gö6976 (inhibits PKC α, β and PKD) or Gö6983 (inhibits PKC α, β, γ, δ or ζ). Treatment of HEK293 or Vero cells with Gö6983 prior to stimulation with TPA or vanadate resulted in strongly reduced phosphorylation of agnoprotein at Ser-11 compared to cells treated with TPA or vanadate alone (compare lane 4

**Fig. 3.** Activation of the PKC and PKD pathways induces phosphorylation of agnoprotein at Ser-11. (A) HEK293 and Vero cells were transfected in 6 wells dishes with 2 μg of pRcCMV-Agno. Serum-starved HEK293 (left panel) and Vero cells (right panel) were vehicle treated or incubated for 30 min with 100 nM TPA, 75 μM sodium pervanadate, 10 ng/ml EGF, or 10 μM Fsk. Phospho-agnoprotein levels were determined by western blot analysis using phospho-specific antibodies that recognize phospho-Ser-11 agnoprotein. In order to verify equal loading, the membrane was stripped and re-incubated with antibodies against agnoprotein. The results are representative for three independent experiments. The intensity of the protein bands in the presented figure was determined by densitometry. The ratio of phosphorylated agnoprotein: non-phosporylated agnoprotein was calculated. The relative ratio in the presence of vehicle or empty vector was arbitrarily set as 1, and the fold induction of phosphorylation was calculated related to this and is presented graphically below the blot. (B) HEK293 and Vero cells were transfected in 6 wells dishes with 2 μg of pRcCMV-Agno and 2 μg empty vector (pEFneo), constitutive active (ca) PKCα-pEFneo, ca PKCε-pEFneo, ca PKCζ-pcDNA3,ca PKD1-pcDNA3 or pSR-Cα expression vector. The serum-starved cells were harvested and whole cell extract were used in immunoblot with phospho-Ser-11 antibodies and the loading was verified with anti-agno-antibodies. The results are representative for three independent experiments. The relative phosphorylation of agnoprotein compared to loading level and vehicle treated cells were evaluated as in A. (C) HEK293 and Vero cells were transfected in 6 wells dishes with 2 μg of pRcCMV-Agno. Serum-starved HEK293 and Vero cells were left untreated or incubated Gö6976 (1 μM) or Gö6983 (1 μM) for 1 h or/and subsequently exposed 30 min to 100 nM TPA or 75 μM sodium pervanadate. Phospho-agnoprotein levels were determined by western blot analysis using phospho-specific antibodies that recognize phospho-serine-11 agnoprotein. In order to verify equal loading, the membrane was stripped and re-incubated with antibodies against agnoprotein. The results are representative for three independent experiments. The relative phosphorylation of agnoprotein compared to loading level and vehicle treated cells were evaluated as in A. (D) Vero cells were transfected with BKV (Dunlop) DNA. 47 h post transfection the cells were exposed to vehicle (DMSO), Gö6983 (1 μM) or GF109203X for 1 h. The cells were subsequently harvested and the cell lysates were used for immunoblot with phospho-Ser-11 specific antibodies. To verify equal loading and blotting of the samples, the membrane was stripped and re-challenged with antibodies against agnoprotein. The result is representative for two independent experiments. The relative phosphorylation of agnoprotein compared to loading level and vehicle treated cells were evaluated as in A.

and 7 with 5 and 8, respectively in the upper panel of Fig. 3C). The levels of total agnoprotein were similar in the samples, indicating that the decreased phosphorylation is not due to reduced protein levels (Fig. 3C, lower panel). The reduced phosphorylation of Ser-11 in presence of Gö6983 indicate that PKC α, β, γ, δ or ζ are involved in mediating the phosphorylation. Presence of Gö6976 prior to stimulation with TPA or vanadate did also reduce phosphorylation of Ser-11 (compare lane 6 to 4 in both panels in Fig. 3C) but less efficient than Gö6983 inhibitor (compare lane 6 to 5). This argues against involvement of PKD in mediating phosphorylation of agnoprotein at Ser-11.

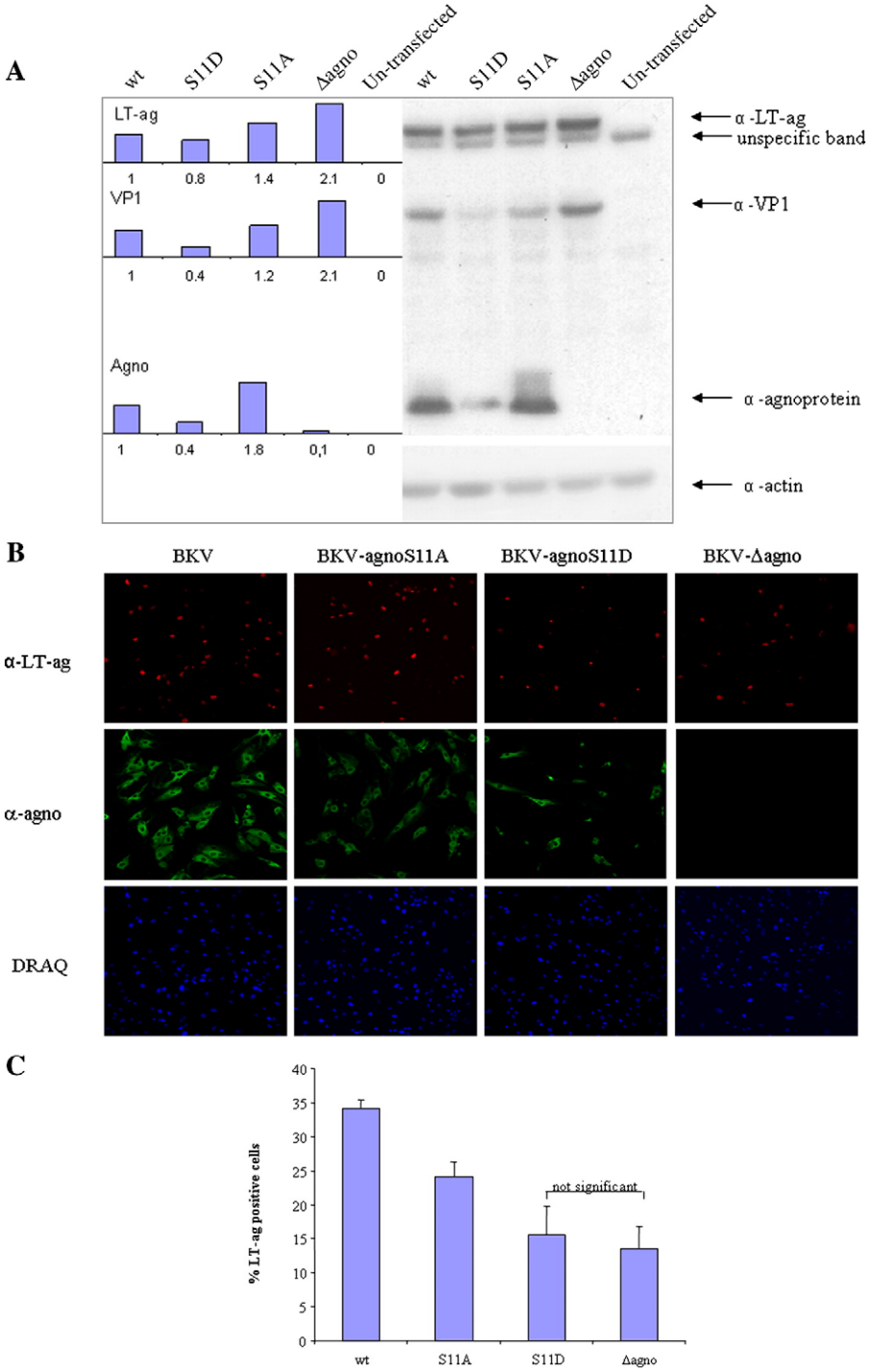



**Fig. 5.** Mutation of Ser-11 influences the ability of viral propagation. (A)Vero cells were transfected in 12 well dishes with 800 ng DNA from BKV wt, BKVagnoS11A, BKVagnoS11D or BKV-Δagno. The cell extracts were harvested 2 days post transfection (dpt), and viral protein expression was assayed by immunoblot with the indicated antibodies. The position of the unspecific band that was also observed in untransfected cells is indicated by an arrow. The results are representative for four independent experiments. Densitometry of the protein bands was performed on the presented blot. The expression of the viral protein was related to actin loading. The relative level of wildtype viral protein was arbitrary set as 1, and the levels of mutant proteins were related according to this (shown in the left panel). (B) The media of Vero cells transfected with DNA from BKV wt or mutants were collected 4 d.p.t and used for infection of HUV-EC-C cells. The HUV-EC-C cells were fixed 5 days after infection, and immunostained with indicated antibodies. The nucleus was stained with DRAQ5. The results are representative for three independent experiments. (C) Percentage LT-ag positive cells in (B) were counted in 4–6 pictures (total of 5–600 cells), and the standard deviation was calculated. The differences in %-LT-ag positive cells between the different BKV variants inclusive wildtype was significant ($p<0.005$) except between BKVagnoS11D and BKVΔagno.

Indeed, depletion of PKD by siRNA treatment did not reduce TPA- or vanadate-induced phospho-Ser-11 levels, emphasizing that PKD did not mediate agnoprotein phosphorylation by these stimuli (results not shown). In conclusion, the stimuli-induced activation of PKC and/or PKD in the presence or absence of inhibitors indicates that PKC, but not PKD, can be involved in the phosphorylation of agnoprotein at Ser-11 *in vivo*.

Finally, we wanted to check whether PKC is involved in phosphorylation of agnoprotein during BKV propagation. We transfected Vero cells with the complete BKV Dunlop genome, and late in the first round of viral life cycle (2 days post transfection), the cells were exposed to vehicle (DMSO), or to the inhibitors Gö6983 or GF109203X. The latter compound inhibits the PKC α, β, γ, ε, η, θ and ζ isoforms. One hour after the addition of inhibitor, the cells were harvested and the phospho-Ser-11 levels of agnoprotein were monitored. First of all, our result shows that agnoprotein was phosphorylated at Ser-11 late in the viral life cycle in BKV-transfected Vero cells (Fig. 3D, lane 1, upper panel). Moreover, the presence of PKC inhibitor Gö6983 as well as GF109203X clearly reduced the phosphorylation of agnoprotein at Ser-11. These results underscore that PKC is involved in mediating phosphorylation of agnoprotein at Ser-11 at this particular time point during viral life cycle (Fig. 3D, upper panel). The presence of comparable levels of agnoprotein in all samples was verified by immunoblotting with antibodies against agnoprotein (Fig. 3D, lower panel).

In conclusion, our findings clearly demonstrate that PKC, but neither PKA nor PKD, can mediate phosphorylation of BKV agnoprotein at Ser-11 *in vivo*.

### *Agno-Ser-11 phosphorylation fluctuates during BKV infection cycle*

The phosphorylation of SV40 LT-ag at residue Thr-124 is the sole post-translational modification required for initiation of replication (Kim et al., 2002 and references therein). The ability of regulating the phosphorylation of this residue is critical for viral propagation, as mutation of T124 in SV40 LT-ag or T125 in JCV LT-ag to alanine results in a replication-defective virus (Schneider and Fanning 1988; Swenson and Frisque, 1995). This clearly reveals that the ability of regulating the phosphorylation state of a protein is highly important. The phosphorylation pattern of a protein may vary in time-dependent manner depending on the activity of cellular signal transduction pathways, which again can be influenced by the state of viral life cycle. Therefore, we wondered whether phosphorylation of agnoprotein at Ser-11 in BKV-transfected- or infected cells is constitutive or fluctuating. Thereto, Vero were transfected with BKV Dunlop DNA. We also infected the human cell line HUV-EC-C, which is highly permissive for BKV (Rinaldo et al., 2005). The cells were harvested after 24 h, and then every hour from 43 and 50 h post transfection (h.p.t) or post infection (h.p.i). The cell lysate was used in an immunoblot with the phospho-Ser-11 specific antibodies (Fig. 4A, upper panel). The expression of agnoprotein was determined by the use of antibodies against agnoprotein (Fig. 4A, middle panel), and loading was verified by use of anti-CREB antibodies (Fig. 4A, lower panel). As can be seen in Fig. 4A, middle lane, agnoprotein is expressed at all time points except 24 h.p.t/h.p.i, while phosphorylation of agnoprotein at Ser- 11 fluctuates in both cell lines (Fig. 4A, upper panel). Similar fluctuation was seen in both transfected Vero cells and infected HUV-EC-C from 33 to 57 h.p.t/h.p.i (results not shown).

The Vero and/or HUV-EC-C cells used above was seeded out one day prior to transfection and/or infection, but were not synchronized further. However, after transfection or infection the early gene products of BK virus will enforce the cells into the S-phase (Moens et al., 2007a,b), which results in a population where the majority of cells is synchronized with the virus in a similar state of the viral life cycle. In order to test this assumption, we wanted to evaluate whether we were able to see fluctuation in phosphorylation of an endogenous protein in a time-dependent manner. CREB is a transcription factor which is regulated by phosphorylation of Ser-133 (Johannessen et al., 2004), and several kinases, including PKC (Xie and Rothstein, 1995), may be involved in phosphorylating this residue. The membrane used in Fig. 4A was stripped and evaluated with phospho-Ser-133 specific antibodies. The ratio phospho-CREB versus CREB was determined and is presented graphically in Fig. 4B. The result reveal that also the phosphorylation pattern of an endogenous protein fluctuates, confirming that most of the cells are relatively synchronized.

### *Mutation of Ser-11 influences viral propagation*

Oscillations in the phosphorylation level of agnoprotein at serine 11 in both Vero and HUV-EC-C suggest a regulatory role of this post-translational modification during the viral propagation. To evaluate the functional consequence of phosphorylation of agnoprotein on BKV propagation, we employed BKV Dunlop strain encoding wildtype agnoprotein, hereafter referred to as BKVwildtype, to create mutants in which serine 11 was mutated to the non-phosphorylable alanine (BKVagnoS11A) or to a phosphorylation mimicking aspartate (BKVagnoS11D). We also generated an agno-deficient BKV Dunlop strain (BKVΔagno) by substituting the start codon with a non-sense codon, and included this agnoprotein-deficient strain in the experiments. This control allowed us to compare the effect of mutations affecting phosphorylation of agnoprotein versus the lack of agnoprotein expression on the viral replication.

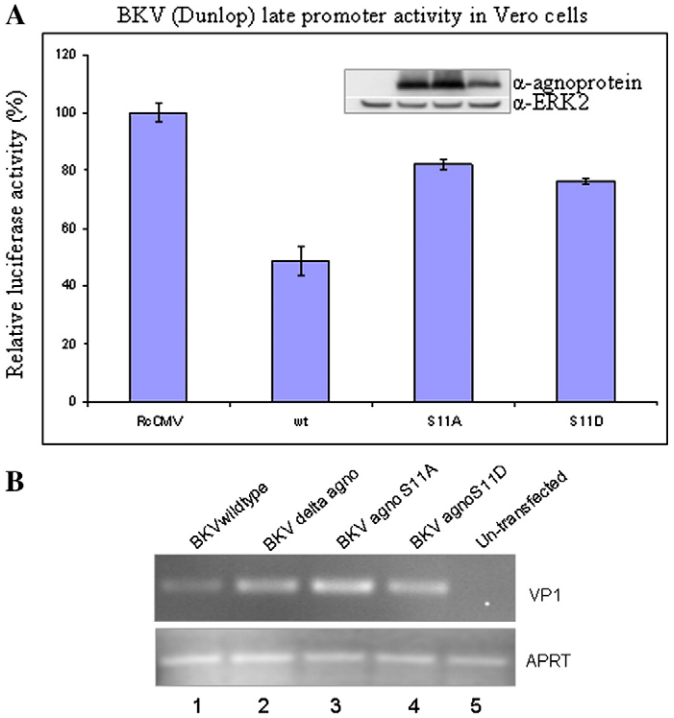



**Fig. 6.** Mutation of Ser-11 reduces the transrepressive effect of agnoprotein. (A) Vero cells were co-transfected in 6 well dishes with 2 μg empty expression vector pRcCMV, pRcCMV-agno, pRcCMV-agnoS11A or pRcCMV-agnoS11D, respectively, and 1 μg luciferase reporter plasmid. The expression of the *luciferase* gene is under control of the late BKV (Dunlop) promoter. The luciferase activity in cell extracts was determined the day after the transfection. The activity in extracts of cells transfected with the empty pRcCMV expression vector was arbitrary set as 100% and the activities in the other extracts were related to this value. The results represent the average of three parallels ± SD and the figure is representative for three independent experiments. Expression of wildtype and mutant forms of agnoprotein was verified by western blot of cell lysates and the loading was controlled by ERK2 antibodies (lower panel). (B) Vero cells were transfected in 12 well dishes with 800 ng DNA from BKV wt, BKVagnoS11A or BKVagnoS11D. Total RNA was extracted 40 h.p.t, and reverse transcribed into cDNA. PCR was performed with specific primers against VP1 or the housekeeping gene APRT. The PCR products were analyzed on an agarose gel in presence of ethidium bromide.

The BKV variants were used to transfect Vero cells. The transfection efficiency among BKV and BKV mutants were similar, as compared by counting cells immunostained with antibodies against LT-ag, related to DRAQ5 stained cells (data not shown). The amount of viral proteins 2 d.p.t. was determined by immunoblot with antibodies against VP1, LT-ag, and agnoprotein. To ensure equal loading, we also monitored the levels of actin in each sample. The strength of the protein bands was evaluated by densitometry, and correlated to loading. The wildtype level of viral protein was arbitrary set as 1, and the values for the mutants were related according to this. The result showing the viral gene expression is presented graphically beside the western blot in Fig. 5A. The BKVΔagno had slightly increased level of LT-ag 2 d.p.t, while BKVagnoS11A and BKVagnoS11D were comparable with BKVwildtype (top band in Fig. 5A and results not shown). An unspecific band, also visible in non-BKV-transfected cell migrated just bellow LT-ag and was present in all samples. The amount of VP1 seemed to be comparable for BKV and BKVagnoS11A in all four parallel experiments (Fig. 5A, and results not shown). In contrast, BKVagnoS11D-transfected cells showed clearly reduced level of VP1, while BKVΔagno clearly had increased VP1 level compared to the other BKV variants in four independent experiments (Fig. 5A and results not shown). The level of agnoprotein also varied among BKV and BKV mutants. BKVagnoS11A expressed more agnoprotein than BKVwildtype (after correction for actin), while the level was robustly reduced in BKVagnoS11D-transfected cells (Fig. 5A and results not shown). As expected, the BKVΔagno variant did not express the agnoprotein. The pattern of viral gene expression was similar 4 and 8 d.p.t (results not shown). These results indicate that BKV mutants have changed viral gene expression compared to wildtype.

In order to compare the amount of released infectious viral particles produced by the cells transfected with wildtype or mutant BKV genomes, media was harvested 4 d.p.t. To ensure that the S11 mutations or the non-sense mutation in Δagno were still present in the genomes of the virus particles shed from the transfected Vero cells, PCR amplification and subsequent sequencing of the BKV DNA sequences present in the collected media was performed. The sequencing results confirmed that the introduced mutations were still present (data not shown). The collected media was then inoculated on HUV-EC-C cells. Counting the number of BKV-positive HUV-EC-C cells will then provide an estimate of the numbers of infectious particles present in the media of BKV DNA-transfected cells (Dugan et al., 2007). The HUV-EC-C cells, which were infected with media obtained from cells containing replicating wildtype or mutant BKV genome, were DRAQ5 stained and immunoassayed with antibodies against LT-ag and agnoprotein 5 d.p.i. (Fig. 5B). The number of infectious particles present in the media from cells transfected with the individual BKV variants was evaluated by counting the number of LT-positive cells among DRAQ5 stained cells and is presented graphically in Fig. 5C. While approximately 35% of the HUV-EC-C cells were infected by media containing BKVwildtype, the number was 25% for media containing BKVagnoS11A (Figs. 5B and C). The media obtained from cells transfected with BKVagnoS11D and BKVΔagno contained a similar number of infectious virions (around 15%), which was significantly less than the amount of infectious particles present in the media of Vero cells transfected with BKVwildtype or BKVagnoS11A DNA (Figs. 5B and C). These results indicate that mutations in the phospho-acceptor site for PKC in agnoprotein affect viral propagation. Moreover, this study shows that BKV lacking agnoprotein is infectious, although with reduced property compared to BKVwildtype. Finally, viral gene expression of BKV- or BKV mutant-infected HUV-EC-C cells was evaluated by immunoblot using antibodies against LT-ag, VP1 and agnoprotein. The results demonstrated that viral gene expression in mutant-infected cells was changed compared to cell infected with BKVwildtype in this cell line, too (results not shown).

In summary, our results noticeably demonstrate that BKV with mutation in phospho-acceptor site Ser-11 hampers viral propagation and results in changes in late viral gene expression compared to wildtype.

### *Influence of Ser-11 phosphorylation on expression of viral proteins*

We found that BKVΔagno had increased level of VP1 compared to BKVwildtype, indicating that expression of agnoprotein reduced the level of VP1 *in vivo* during BKVwildtype infection (compare BKVwildtype with BKVΔagno in Fig. 5A). Moreover, the BKVagnoS11D had reduced level of VP1 and agnoprotein, while BKVagnoS11A showed an increased level of agnoprotein compared to BKVwildtype (compare agnoprotein and VP1 levels for BKVwildtype-, and BKVagnoS11A-,

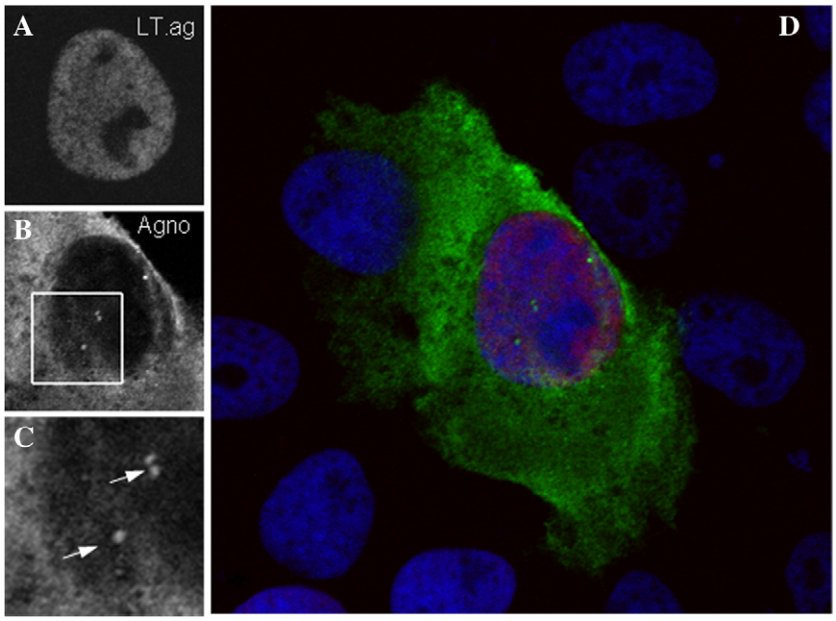



**Fig. 7.** Agnoprotein localization in the cell. Confocal microscopy of Vero cells transfected with 200 ng wt BKV DNA in 8 well Chamber slides. The cells were fixated 2 d.p.t. and immunostained with antibodies. (A) The LT-ag staining of a BKV-transfected cell (B) Agnoprotein-staining of a BKV-transfected cell (C) Enlarged section of the agnoprotein staining seen in (B) (D) Stack overlay labeled with agnoprotein (green), LT-ag (red) and DRAQ5 (blue).

BKVagnoS11D-transfected cells in Fig. 5A). This observation suggests that agnoprotein and mutations that interfere with *bona fide* phosphorylation of the agnoprotein influence on late viral gene expression. The gene expression is the sum of transcription, post-transcriptional, translational and post-translational process (see Discussion). We decided to evaluate the effect of agnoprotein and its variants with a mutation in the phospho-Ser-11 site on the activity of the late Dunlop promoter in order to test the effect on transcription. Thereto, Vero cells were co-transfected with an expression plasmid encoding wildtype agnoprotein, agnoproteinS11A or agnoproteinS11D and a reporter plasmid with the *luciferase* gene driven by the late promoter of BKV Dunlop. The presence of wildtype agnoprotein repressed transactivation of the late BKV promoter by approximately 50%, while only a slight repression was monitored with agnoproteinS11A (Fig. 6A), suggesting that mutation into non-phosphorylable alanine almost abolishes the repressive effect of agnoprotein. If so, one might assume that a phospho-mimicking mutant agnoproteinS11D should restore the transrepressive effect but surprisingly agnoproteinS11D showed similar transrepressive effect as agnoproteinS11A. The expression level of agnoprotein in the cell lysates used for luciferase measurements was then checked by immunoblotting with antibodies specific for agnoprotein and the loading was controlled by antibodies against ERK2 (Fig. 6A upper right corner). The result revealed that the expression of agnoS11D clearly was reduced compared to wt and agnoproteinS11A, which may explain the lack of influence on the reporter. As a second experimental strategy, we next wanted to evaluate whether the VP1 transcript levels varied among the BKV variants. Total RNA was therefore harvested 40 h post transfection and a semi-quantitative RT PCR was performed to determine the mRNA levels of VP1. BKVagnoS11A-transfected cells display increased VP1 transcript levels compared to wildtype, which is in agreement with the reporter assay shown in Fig. 6A. Similarly, the BKVagnoS11D-transfected cells shows slightly increased VP1 mRNA level compared to wildtype, but clearly less than BKVagnoS11A-transfected cells, again in agreement with the reporter assays. Also BKVΔagno-transfected cells shows clearly increased level of VP1 transcript, confirming the transrepressive effect of agnoprotein seen in Fig. 6A. This may also suggest that the increase of VP1 protein levels seen for BKVΔagno in Fig. 5A indeed could be a consequence of inhibitory function of agnoprotein at viral late gene transcription.

Phosphorylation may affect the intrinsic repression potential of agnoprotein. Alternatively or in addition, phosphorylation may affect the subcellular localization of agnoprotein and hence its ability to influence promoter activity. Indeed, agnoprotein resides mainly in the cytoplasm, but the agnoprotein of JCV and SV40 has also been found in the nucleus (Khalili et al., 2005). Okada et al. (2001) reported that phosphorylation of JCV agnoprotein altered its localization from perinuclear region into the nucleus. Therefore, we wanted to evaluate whether the differences in transrepressive property of wt and mutated agnoprotein could be explained by phoshorylation-dependent subcellular redistribution of the agnoprotein. Thereto, Vero cells were transfected with BKV, BKVagnoS11A or BKVagnoS11D. Two days post transfection, the cells were fixated and immunostained with antibodies against agnoprotein and LT-ag, while the nucleus was stained with DRAQ5. At least 50 cells of each variant were examined by confocal microscopy, where the nucleus was divided into several sections. As seen in Fig. 7, a small fraction of BKV agnoprotein can be detected in the nucleus. This was seen in approximately 10% of the cells examined. The amount of cells containing agnoprotein in nucleus did not differ significantly among BKV, BKVagnoS11A and BKVagnoS11D (results not shown). Therefore, we believe that the differences in the transrepressive effect of agnoprotein versus agnoproteinS11A on BKV late promoter (Figs. 6A and B) is not due to phosphorylation-dependent changes in subcellular localization, but rather originates from phosphorylation-triggered differences in the intrinsic repression function of the protein.

*Mutation of Ser-11 influences the stability of BKV agnoprotein*

The stability of several proteins has been shown to be regulated by phosphorylation (Whitmarsh and Davis, 2000). This prompted us to test whether the variations seen in the amount of agnoprotein in Vero cells transfected with BKVwildtype compared to BKVagnoS11A and BKVagnoS11D at day 2 (Fig. 5A) and in the lysates used in the luciferase assays (Fig. 6A) could be due to differences in the phosphorylation-regulated stability of the protein. Thereto, we transfected Vero cells with expression plasmids for agnoprotein or agnoprotein mutated in Ser-11 residue. The protein synthesis in the cells was inhibited by the addition of cycloheximide and the level of agnoprotein was evaluated by immunoblot with antibodies against agnoprotein at 0, 2, 4, 6, 8 and 12 h after the administering of cycloheximide. As depicted in Fig. 8, the level of agnoproteinS11D was clearly reduced after 6 h, while the level of wt agnoprotein was only slightly decreased at this time point. The S11A mutation seemed to stabilize the agnoprotein because no obvious

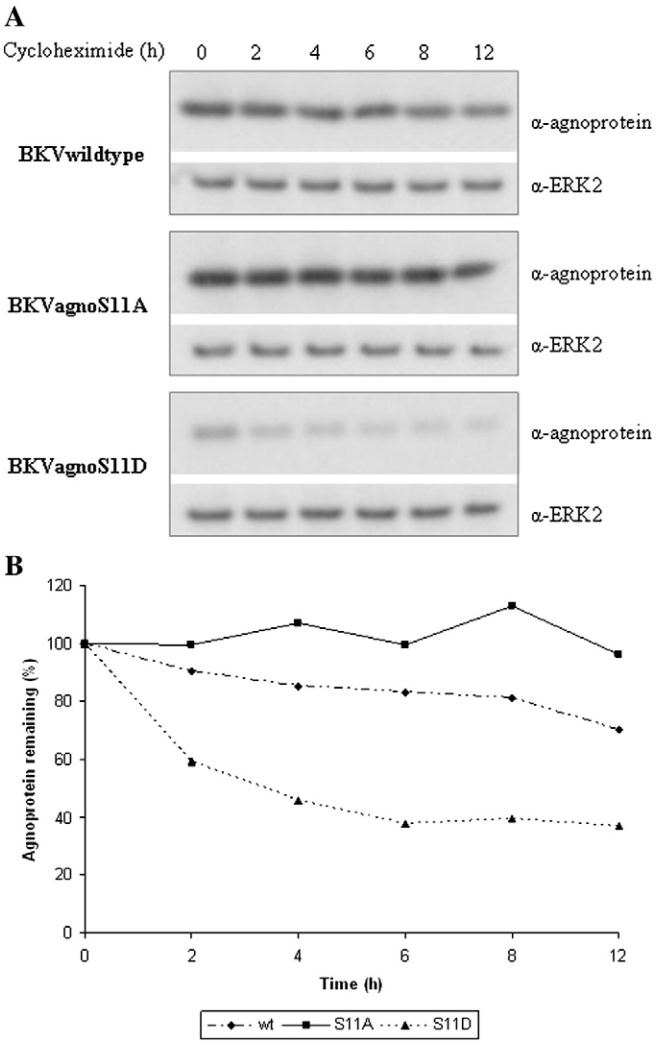



**Fig. 8.** Mutation of Ser-11 influences the stability of agnoprotein. (A)Vero cells were transfected with pRcCMV-agno, pRcCMV-agnoS11A or pRcCMV-agnoS11D. Cycloheximide (100 μg/ml) was added and the cells were harvested 0, 2, 4, 6, 8 and 12 h after the addition of protein synthesis inhibitor. An immunoblot was performed using antibodies against agnoprotein (upper lane). To verify equal loading and blotting of the samples, the membrane was stripped and re-incubated with anti-ERK2 antibodies (lower panel). The results are representative for three independent experiments. (B) The relative levels of wt agnoprotein and mutant protein were determined by densitometric scanning of the signals obtained after immunoblotting. The strength of the signal obtained for the protein level assays before the addition of cycloheximide ($t=0$) was arbitrary set as 100%.

reduction in S11A agnoprotein levels was monitored even after 12 h incubation with cycloheximide. In conclusion, phospho-mimicking mutations trigger agnoprotein destabilization, while the non-phosphorylable alanine seems to increase the stability of agnoprotein. This might partly explain the changed level of agnoprotein in the BKV- or BKV mutant-transfected cells in Fig. 5A.

## Discussion

In this study we show that BKV agnoprotein can be phosphorylated at Ser-11 by PKA, PKC and PKD, while the two latter enzymes also can phosphorylate Ser-7 and Thr-21 *in vitro*. Moreover, we demonstrate that PKC can be involved in Ser-11 phosphorylation of BKV agnoprotein *in vivo*. The phosphorylation pattern of serine 11 of BKV agnoprotein fluctuates during viral propagation, suggesting an important regulatory role. Indeed, our study reveals that mutation of Ser-11 into alanine or aspartate results in disturbed propagation with reduced production of infectious viral particle. Moreover, the BKV variants with mutations in the phospho-acceptor site Ser-11 of agnoprotein show a changed late gene expression compared to the wildtype in the two tested cell lines. The reduced transcriptional repression activity of the agnoproteinS11A and agnoproteinS11D mutants on the BKV promoter is partly due to reduced intrinsic repression potentials of the mutants compared to wildtype agnoprotein and to changed stability of agnoprotein variants.

The agnoproteins of BKV, JCV and SV40 are phospho-proteins (Khalili et al., 2005). One study has suggested the involvement of PKA in phosphorylation-mediated regulation of JCV agnoprotein (Okada et al., 2001). In another study, PKC was added to cell lysate, containing phosphatase-treated JCV agnoprotein, JCV agnoproteinT21A, JCV agnoproteinS7A/S11/A or JCV agnoproteinS7A/S11A/T21A. The result indicated that all three sites in JCV agnoprotein could be phosphorylated by PKC (Sariyer et al., 2006). In our study, we have found that while PKA, PKC and PKD all can phosphorylate BKV agnoprotein *in vitro*, only PKC seems to be involved in phosphorylation of BKV agnoprotein Ser-11 *in vivo* as evaluated in cell culture system in the presence of stimuli, inhibitors, or a constitutive active kinase variant. Moreover, we also found that inhibition of the PKC pathway resulted in reduced phosphorylation of agnoprotein at Ser-11 during viral propagation 48 h post transfection. This clearly shows that PKC indeed is able to phosphorylate BKV agnoprotein at Ser-11 *in vivo* during viral infection. However, the viral life cycle occurs within a living cell, where the different signaling pathways, involving numerous protein kinases, may continuously be switched on and off depending on environmental- and virus induced-stimuli. This may be reflected by the fluctuation of the Ser-11 during viral propagation (Fig. 4A). So even though PKC can mediate *in vivo* phosphorylation at agnoprotein Ser-11 at 48 h.p.t., other kinase(s) may be involved at other time points during viral life cycle. Moreover, the activity and/or presence of phosphatases may influence the phosphorylation of agnoprotein. Indeed, for JC virus it was recently shown st-ag is involved in the dephosphorylation of agnoprotein (Sariyer et al., 2008). In our study, we also found that the phospho-acceptor sites Ser-7 and Thr-21 in BKV agnoprotein may be phosphorylated by PKC and PKD *in vitro*. However, it remains to be established whether PKC and/or PKD are involved in the phosphorylation of these sites *in vivo*.

Comparisons of protein levels in Vero cells transfected with BKV- or BKV mutants showed that BKVagnoS11A, BKVagnoS11D and BKVΔagno had a changed viral late gene expression compared to the wildtype (Fig. 5A). Gene expression is the sum of several processes and includes among others: transactivation of the promoter, regulation of RNA transcripts, translation efficiency, and stability of the protein which all will be discussed regarding our result in the following.

Several studies in JCV have suggested that agnoprotein may be involved in viral gene transcription. In one study, JCV Mad1 or JCV Mad1Δagno was used to transfect the susceptible neuroblastoma cell line IMR-32. The viral gene transcription of LT-ag, VP1 and agnoprotein were evaluated by RT PCR, and the authors conclude that the absence of agnoprotein had a negative influence on viral transcription (Okada et al., 2001). Here we found that agnoprotein with serine-11 replaced by alanine has reduced transrepressive effect on the late viral promoter (Fig. 6). The agnoproteinS11D did not influence the late transcription in Vero cells which may be due to its rapid degradation (Fig. 6A upper right corner and Fig. 8). Our results suggest that phosphorylation of agnoprotein may influence the intrinsic repressive property of the protein. The exact mechanism for agnoprotein-mediated repression is not known, but as we found BKV agnoprotein in nucleus of 10% of the cells examined (Fig. 7), it may involve phosphorylation-regulated interaction with nuclear proteins. Indeed, studies with reporter constructs revealed that JCV agnoprotein can interact with LT-ag and the cellular transcription factor YB-1, and suppress JCV LT-ag-mediated transactivation of late reporter and YB-1-mediated transactivation of both early and late reporter in the human cell line U-87MG (Safak and Khalili, 2001; Safak et al., 2001; Safak et al., 2002). Our preliminary results suggest that BKV LT-ag and agnoprotein can interact *in vivo* (results not shown), however, whether this interaction is regulated by phosphorylation as well as the biological consequence of this association remains to be investigated.

The level of VP1 varies among BKV-transfected cells, and the highest level is observed in cells transfected with BKVΔagno (Fig. 5A). Agnoprotein and VP1 are both assumed to be on the 16S transcript (Cole and Conzen, 2001) and the ratio VP1 versus agnoprotein in Vero cells transfected with BKV or BKV variants is not constant (Fig. 5A). This argues against transactivation as the *only* regulatory mechanism for the changed viral gene expression caused by the mutations in agnoprotein. The change in viral gene expression as seen in Fig. 5A might also be due to distinct abilities of agnoprotein variants to influence the RNA levels. For SV40, an early study revealed that cells infected with agnoprotein-deficient mutants possessed increased transcript levels of early mRNA and of the late 16S as well as the 19S transcripts compared to wildtype infected cells. The rate of transcription did not differ among wildtype and the agnoprotein-lacking mutants, indicating that the observed increase in amount of transcripts was due to some kind of post-transcriptional regulation (Resnick and Shenk, 1986). BKV also produces 16S and 19S transcript (Manaker et al., 1979; Moens et al., 1994), but whether BKV agnoprotein in a phosphorylation-regulated manner influences the mRNA of late genes remains to be established. Therefore, we cannot rule out that the increased VP1 protein levels in BKVΔagno and of agnoprotein in BKVagnoS11A (Fig. 5A) result from changes at the post-transcriptional level in addition to reduced transrepressive effect in the case of agnoproteinS11A (Fig. 6A and B).

Polyomavirus transcripts are polycistronic, which is uncommon in eukaryotic cell. Translation of mRNA usually starts with ribosomes that enter at the 5′ end of the transcript, and then scan the mRNA for the start codon. The efficiency of initiation of translation at this start codon depends on its sequence context and the distance to 5′ end (Kozak, 2007). Indeed, the efficiency of translational initiation of agnoprotein at AUG has been shown to depend upon the distance to the 5′ end (Sedman et al., 1990). Agnoprotein may thus be translated with various degree of efficiency depending on the length of the untranslated 5′ end of the transcript. In both 16S and 19S mRNAs, the agnoprotein start codon precedes VP1, and VP2 and VP3 AUGs, respectively (Dabrowski and Alwine, 1988). The presence of another start codon may affect translation from the downstream start codon as was shown for SV40 late mRNAs, where mutation of the start codon of SV40 agnoprotein resulted in increased VP1 expression (Grass and Manley, 1987; Perez et al., 1987). In our study, we also observed increased VP1 production with BKVΔagno, in which the start codon of agnogene was mutated into TTG. This suggests that improved accessibility of the VP1 start codon by the ribosomes may result in augmented VP1 production.

The stability of a protein may also influence its expression level. Sariyer et al. (2006) compared the expression of agnoprotein versus agnoprotein T21A, S7A/S11A and S7A/S11A/T21A during viral propagation. They found that the JCV agnoprotein with mutations in the phospho-acceptor sites was expressed to a higher level than wildtype during the infection cycle. The increase of mutant agnoprotein expression was, however, not due to elevated turnover rates (Sariyer et al., 2006). We compared the stability of BKV agnoprotein versus BKV agnoproteinS11A or BKV agnoproteinS11D and found that the latter is less stable than wildtype, while agnoproteinS11A is more stable (Fig. 8). This may partly explain the changes in mutant agnoprotein levels compared to the wildtype protein observed in BKV-transfected cells (Fig. 5A). It is also tempting to speculate that the mutation in agnoprotein influences the VP1 stability, thereby causing the variable VP1 levels seen among the different BKV variants in Fig. 5A. Studies in SV40 have shown that agnoprotein may be involved in localization of VP1, suggesting an interaction between the two viral proteins (Carswell and Alwine, 1986). Moreover, one study revealed that missense mutations in the *VP1* gene of SV40 can compensate for defects caused by deletions in the viral agnogene (Barkan et al., 1987), confirming putative interaction between the two viral proteins. Another early study in SV40 demonstrated that the rate of expression of VP1 protein is similar between wt SV40 and agnoprotein-deficient SV40. However, under long-term labeling condition where labeled VP1 was representative for steady state VP1 level, the authors consistently observed 50% reduction in VP1 accumulation in mutant versus wildtype infected cells. The authors suggest that VP1 is less stable in absence of agnoprotein (Resnick and Shenk, 1986). Moreover, in another early study second-site revertants were isolated of a partially defective VP1 mutant of simian virus 40. The suppressing mutation in each of these pseudorevertants was mapped to the viral agnogene. Of six independently isolated pseudorevertants, all had a missense mutation in serine at position 7, 11 or 17 in the agnoprotein by a hydrophobic amino acid. Their results suggest that the agnoprotein interacts in a specific way with VP1 during the late stages of viral development and in the discussion the authors speculated that agnoprotein and VP1 interaction may be regulated by phosphorylation (Margolskee and Nathans, 1983). However, despite the high amino acid identity between SV40 and BKV agnoprotein (67%) (Moens et al., 2007a,b), an interaction between BKV agnoprotein and VP1 and a possible involvement of protein phosphorylation remains to be established.

Our BKVagnoS11A, BKVagnoS11D and BKVΔagno mutants still sustained the viral infection cycle, even though at a lower the efficiency compared to BKVwildtype. This seems in contrast with JCV where the agnomutants T21A, S7A/S11A, and S7A/S11A/T21A were unable to produce infectious virions, because these mutations interfered with genomic encapsulation (Sariyer et al., 2006). However, we have not investigated the viral replication efficiency of the corresponding BKV mutants. Phosphorylation of the conserved phospho-acceptor sites in BKV and JCV agnoprotein may have different effects on the functions of the proteins. Furthermore, BKV and JCV display distinct cell tropism and hence agnoprotein function and regulation of these two viruses may be cell-specific and explain the differences in viral propagation between phospho-acceptor agnoprotein mutants of JCV and BKV.

Our results suggest that reduced propagation of the BKV mutants compared to wildtype is partially due to disturbed viral gene expression. Nuclear egress of JCV viral particles was shown to require interaction of agnoprotein with HP1α (Okada et al., 2005). Whether BKV agnoprotein interacts with HP1α and whether this interaction is regulated by phosphorylation remains unknown, but the 18 first amino acids of JCV agnoprotein, which are absolutely necessary for the association with HP1α (Okada et al., 2005), are conserved in BKV agnoprotein (Moens et al., 2007a,b). Moreover, phosphorylation of histone H1 by CDK regulates its binding to HP1α (Hale et al., 2006), and it is tempting to speculate in a similar regulation of agnoprotein–HP1α interaction.

In summary, we have shown that BKV agnoprotein can be phosphorylated at Ser-11 by PKC *in vivo*. The phosphorylation pattern at this site oscillates during viral infection cycle and mutations of this residue reduced viral propagation. At least two mechanisms seem to contribute to perturbed propagation of the agnoprotein serine-11 mutants: altered intrinsic transrepression function as well as stability of the protein. Our results suggest that targeting PKC pathway by specific inhibitors may be a putative for therapy to moderate BKV infection.

## Materials and methods

### *Materials*

12-*O*-tetradecanoylphorbol-13-acetate (TPA), sodium orthovanadate, epidermal growth factor (EGF) and forskolin (fsk) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thrombin was obtained from Amersham Biosciences. Foetal Bovine Serum (FBS) and cell culture medium were obtained from Invitrogen (Carlsbad, CA, USA). CDP-star and MagicMark western standard were from Applied Biosystems (CA, USA) and Invitrogen Life Technologies (Oslo, Norway), respectively. Antibodies against agnoprotein were described previously (Rinaldo, Traavik et al., 1998). Phospho-Ser-11 specific antibodies were ordered from Eurogentec, which prepared affinity-purified antibodies from serum of rabbits immunized with KLH-conjugated CLSRQApSVKVGT. CREB and phospho-CREB Ser-133 antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA), while the antibodies for ERK2 and actin were from Santa Cruz and Sigma-Aldrich, respectively. SV40 LT-ag AB-2 was from Calbiochem, while antibodies for BKV VP1 has been described previously (Grinde et al., 2007). Gö 6976 and Gö 6983 were from Calbiochem (CA, USA), while GF109203X was from Alexis. Alkaline phosphatase conjugated anti-rabbit antibody was purchased from DAKO (Denmark). Activated PKD1 was from Upstate (Medprobe, Oslo, Norway), while the catalytic Cα subunit of PKA and PKC (P0329) were purchased from Sigma-Aldrich.

### *Cell lines*

HEK293 (ATCC CRL-1573), Vero (ATCC CCL81) and HUV-EC-C (ATCC CRL1730) were all purchased from American Type Culture Collection and were maintained according to the instructions by the manufacturer. The stimulus pervanadate was freshly prepared for each experiment according to (Gordon, 1991).

### *Plasmid constructs and mutant viruses*

The empty expression vector pEFneo and the plasmids containing the coding sequences for PKCα A/C, PKCδ A/C and PKCε A/C were a kind gift from Dr. G. Baier (Von Essem et al., 2006). PKCζA119E was kindly provided by Dr. T. Johansen (Perander et al., 2001), while pSR and the plasmid pSR Cα, containing the catalytic Cα subunit from PKA, were a kind gift from Dr. T. Jahnsen and Dr. K. Taskén (Foss et al., 1994). The plasmids pcDNA3.1 and pGEX-4T-1 were purchased from Invitrogen and GE Healthcare, respectively. Constitutive active PKD (744/748E/E mutant) and pRcCMV-agno have been described previously (Johannessen et al., 2007a,b; Rinaldo et al., 1998). pRcCMV-agnoS11A and pRcCMV-agnoS11D were created by site-directed mutagenesis by use of pRcCMV-agno as a template. An XbaI site was created into pGEX-4T-1 generating pGEX-4T-1X by site-directed mutagenesis. Then, EcoRI and XbaI were used to excise the agno coding sequences from pRcCMV-agno, and the agno-fragment was cloned into the pGEX-4T-1X vector, resulting in pGEX-4T-1X-agno. pGEX-4T-1X-agno was used as a template in site-directed mutagenesis in order to create pGEX-4T-1X-agnoS7A, pGEX-4T-1X-agnoS11A, pGEX-4T-1X-agnoT21A, pGEX-4T-1X-agnoS62A, pGEX-4T-1X-agnoS7A/S11A, pGEX-4T-1X-agnoS11A/T21A, pGEX-4T-1X-agnoS7A/T21A, pGEX-4T-1X-agnoS7A/S11A/T21A. The plasmid p34-2 was obtained from ATTC (cat. no. 45025). To minimize the risk for unwanted mutation during PCR-based site-directed

mutagenesis, we created a plasmid with part of BKV genome encompassing the region/sequences encoding agnoprotein. The approximately 500 bp NdeI–PciI fragment of digested p34-2 was ligated into NdeI–PciI digested pUC18, creating the plasmid agno-pUC18. Agno-pUC18 was used for site-directed mutagenesis in order to convert agno-Ser-11 into alanine (agnoS11A-pUC18) or aspartic acid (agnoS11D-pUC18) and to convert the start codon into TTG resulting in plasmid named agno-puc18 Δagno. To be able to reintroduce the mutated 500 bp NdeI and PciI fragment into the BKV genome, we needed to go through an intermediate step with a larger part of the BKV genome in a modified pUC18 vector (pUC18m). pUC18m, lacked NdeI and PciI sites and contained unique BglII and MfeI sites, and was created by NdeI and PciI digestion of pUC18 followed by ligation of a synthesized oligo created by annealing 5′-tac gcc atg cat tgt tag atc tag agt cca att gca cct taa gat gc-3′ and 5′-cat ggc atc tta agg tgc aat tgg act cta gat cta aca atg cat ggc g-3′. p34-2 was thereafter digested with BglII and MfeI and the approximately 1700 bp BglII–MfeI BKV fragment was ligated into pUC18m resulting in the plasmid named modified pUC18-BKV. Into this modified pUC18-BKV were the agnoS11A-puc18, agnoS11D-puc18 and Δagno-puc18 inserted by digestion with NdeI and PciI and ligated into NdeI and PciI digested modified pUC18-BKV. In the last step, the modified pUC18-BKV with mutated insertions, was digested with SacI, and the resulting 500 bp DNA fragment containing part of agnogene was used to replace the same fragment in partial digested p34-2 backbone, according to the cassette model described previously (Johnsen et al., 1995). pGL3-basic was purchased from Promega. pGL-TCR-EL was created by amplifying the transcription control region (TCR) from p34-2 by use of NCCR.F (5′-cta agc ttt tgc aaa aat tgc aaa aga ata gg-3′) and NCCR.R (5′-gta agc ttg gcc ttt gtc cag ttt aac tg-3) primers. The PCR product was digested with HindIII and ligated into pGL3-basic. The orientation of TCR was determined by sequencing. Purified BKV Dunlop was a kind gift from Dr. C.H. Rinaldo (Rinaldo et al., 1998).

*Site-directed mutagenesis*

All PCR-based site-directed mutagenesis were performed using Stratagene Quickchange according to the instructions of the manufacturer (Stratagene, La Jolla, CA, USA). The complementary primer sets for the generation of mutant plasmids were as follows (only one strand is shown): XbaI-primer: 5′-gga att ccc ggg tcg tct aga gcg gcc gca tc-3′; S7A: 5′-ctg cgc cag ctg gca cga caa gct tca g-3′; S11A: 5′-ca cga caa gct gca gtg aaa gtt ggt aaa acc-3′; S11D: 5′-ca cga caa gct gac gtc aaa gtt ggt aaa acc-3′; T21A: 5′-cct gga ctg gag caa aaa aaa gag ctc aga gg-3′; S62A: 5′-gct gta aaa gac gct gta aaa gac tcc-3′; Δagno: 5′-ctg gac aaa ggc ctt ggt tct gcg cca gct g-3′. All mutations were verified by sequencing.

*Sequencing*

Cycle sequencing was performed using the Big Dye sequencing kit version 3.1 (Perkin-Elmer-Tropix). Sequencing reactions were analyzed on an ABI377 Prism Sequencer (Perkin-Elmer).

*In vitro kinase assay*

*In vitro* kinase assay was performed as outlined before (Van Lint et al., 1998). GST fusion proteins were purified from *Escherichia coli* BL21 extracts using glutathione–agarose beads, and the agnoprotein moiety was removed from the GST-beads by use of thrombin according to the instructions of the manufacturer.

*Transient transfection*

Transfection studies with expression plasmids in HEK293 and Vero cells were done using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Luciferase assays were as previously described (Johannessen et al., 2004). Before transfection of the BKV genome, the genome was BamHI digested out of the vector and re-ligated with T4-ligase giving a pool with re-ligated BKV genome and re-ligated pBR322 vector.

*Infections*

HUV-EC-C cells were treated with virus containing media for 2 h at 37 °C, followed by addition of fresh media. Four days later, the cells were either PFA fixated for immunofluorescence analyses, or cell extracts were harvested and used for immunoblot analyses.

*Immunofluorescence analyses*

Cells were rinsed twice with phosphate-buffered saline (PBS) and fixed for 10 min with 4% formaldehyde. Next, the cells were washed twice with PBS and then permeabilized for 10 min with 100% methanol (−20°) for 10 min, and washed twice with cold PBS prior to blocking for 30 min with 3% goat serum at room temperature. Viral proteins were recognized by agno-serum (1:600) and SV40 AB-2 LT-ag 1:100), and stained with secondary antibodies Alexa Fluor 568 (red) to detect LT-ag and Alexa Fluor 488 (green) to detect agnoprotein. The cells were then washed with PBS and examined using confocal laser-scanning Zeiss LSM 510 META and Leica SP5 microscopes. Cell nuclei were visualized by DRAQ5 staining (Biostatus Ltd., Leicestershire, UK).

*Immunoblot analysis*

Immunoblot analyses were performed on *in vitro* phosphorylated proteins or extracts derived from cells as described previously (Johannessen et al., 2007a,b). The densitometric analysis of western blot was accomplished using Multi Gauge Ver3.0 (Fujifilm Life Science, Japan). The measurements were corrected for background signal and normalized to loading control.

*RNA isolation and reverse transcriptase polymerase chain reaction (RT PCR)*

cDNA was made and subjected to PCR. The primers for APRT and the PCR conditions have been previously described (Johannessen et al., 2004). To amplify VP1 cDNA, the following primers were used: 5′-CTTTGCTGTAGGTGGAGAACC C-3′ (forward) and 5′-CTCCTGTGAAA-GTCCCAAAATAC-3′ (reverse). The PCR cycling conditions were 20 times 94 °C for 30 s, 65 °C for 30 s, and 72°C for 2 min s. The VP1 primers were kindly provided by Dr. Christine H Rinaldo, University Hospital of North Norway, Tromsø.

*Statistical analysis*

Student's *t*-test and differences with $p$-values$<0.05$ were considered as significant.

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